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Heat shock proteins (hsps) are induced in cells in response to environmental stresses. It has been shown that some breast cancer patients express high levels of hsp27, which may augment the aggressiveness of these tumors and make them more resistant to treatment. The research funded by this fellowship is directed at understanding the regulation of hsp27 toward the development of a useful therapy for inhibiting breast cancer progression.

In our first year progress report sent for review in October of 1996, we demonstrated substantial progress on Specific Aim 1--the examination of the regulatory mechanisms controlling the expression of hsp27 in breast cancer cells. Therefore, we will focus upon our progress on Specific Aim 2 in this current report. Specific Aim 2 involves the identification of genes whose expression is associated with hsp27 effects on cellular proliferation, metastatic behavior, and drug resistance. We have applied the technique of differential display analysis to isolate 11 candidate cDNA's which were modulated as a result of hsp27 overexpression. Furthermore, we have determined that hsp27's effect on drug resistance involves the inhibition of drug-induced apoptosis. Concomittant with this change is the the production of matrix metalloproteinase 9 in hsp27-overexpressing cells, a finding which might be associated with hsp27's newly discovered role in the metastatic behavior of breast cancer cells. We are currently focusing our efforts on identifying the specific pathways involved.

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Suzanne Joyce 3/13/97

PI - Signature Date

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MAIN REPORT:

ABSTRACT:

Heat shock proteins (hsps) are induced in cells in response to environmental stresses. It has been shown that some breast cancer patients express high levels of hsp27, which may augment the aggressiveness of these tumors and make them more resistant to treatment. The research funded by this fellowship is directed at understanding the regulation of hsp27 toward the development of a useful therapy for inhibiting breast cancer progression.

In our first year progress report sent for review in October of 1996, we demonstrated substantial progress on Specific Aim 1--the examination of the regulatory mechanisms controlling the expression of hsp27 in breast cancer cells. Therefore, we will focus upon our progress on Specific Aim 2 in this current report. Specific Aim 2 involves the identification of genes whose expression is associated with hsp27 effects on cellular proliferation, metastatic behavior, and drug resistance. We have applied the technique of differential display analysis to isolate 11 candidate cDNA's which were modulated as a result of hsp27 overexpression. Furthermore, we have determined that hsp27's effect on drug resistance involves the inhibition of drug-induced apoptosis. Concomittant with this change is the the production of matrix metalloproteinase 9 in hsp27-overexpressing cells, a finding which might be associated with hsp27's newly discovered role in the metastatic behavior of breast cancer cells. We are currently focusing our efforts on identifying the specific pathways involved.

INTRODUCTION:

Heat shock proteins (hsps) are induced by a variety of environmental and physiological stresses (1), and the small molecular weight hsp27 is often found at high

levels in human breast tumors (2). We have demonstrated that hsp27 is involved in regulating the growth of breast cancer cells as well as the development of drug resistance in these cells (3). Recently, we have also discovered a role for hsp27 in the invasion and metastatic behavior of breast cancer cells (4). Based on these findings, we hypothesize that hsp27 plays a pivotal role in breast cancer progression, and have focused upon its regulation, interring with its expression, and understanding the cellular pathways through which it exerts its effects on breast cancer cells.

In this fellowship we have proposed the following Specific Aims:

1. To examine the regulatory mechanisms underlying the expression of hsp27 in breast cancer cell lines and human breast tumors. (Years 1-3)
2. To identify genes whose expression is associated with hsp27 effects on proliferation and drug resistance.. (Year 3)
3. To target positive and negative hsp27 transcriptional regulatory factors identified in Aims 1 and 2 to interfere with hsp27 expression. (Years 2-3)

BODY:

We have made excellent progress in the second year of our investigation focusing predominantly upon Specific Aim 2 and are ahead of our original schedule in the grant. We have elected to utilize the sensitive technique of differential display (DD) (6) to identify new gene products which are associated with the overexpression of hsp27 in human breast cancer cells. The DD technique is a powerful way to evaluate gene expression in cells that have been manipulated to express specific genes. We first put a considerable effort into improving the sensitivity and specificity of the DD technique and published modifications to the general method in BioTechniques (7). Using this modified DD method, we then began to evaluate differential gene expression in MDA-MB-231 breast cancer cells constitutively overexpressing hsp27. Our previous studies had utilized an inducible

expression vector for hsp27 (3), however we felt that the heavy metals required for induction of this hsp27 expression vector might confound our results. So we have generated MDA-MB-231 cells stably overexpressing hsp27 under the control of a constitutive cytomegalovirus promoter. Western blot analysis of hsp27 expression in two control, vector-alone transfected clones (controls 1 and 2) and three hsp27 transfectants (clones 19, 12(2), and 41) is shown in Fig. 1. The range of hsp27 overexpression varied from 2 to 5-fold in the hsp27 transfectants as compared to the control-transfected cells when the immunoblots were evaluated by densitometric scanning. We have now applied DD using a combination of twenty-four 5' arbitrary primers and three 3'anchored primers. From this initial series we have identified 11 cDNA's which are differentially expressed in the hsp27-overexpressing tranfectants. We have focused on two different patterns of expression, the first of which is shown in Fig. 2. Two control-tranfected clones (labeled C) were run alongside two of the transfected hsp27 clones 19 and 12(2) (labeled T). A control lane omitting the RT (-RT) was also included to exclude the amplification of any remaining genomic DNA in the RNA preparations. A molecular weight marker was also run on the gels; the sizes are shown in bp. Fig. 2 exemplifies that we have identified cDNA's which are induced by hsp27 overexpression, and a large cDNA of approximately 480 bp is marked with an arrow in this figure. We have identified eight cDNA's which are induced by hsp27 overexpression in MDA-MB-231 breast cancer cells.

The second pattern of cDNA expression which we have focused upon is that shown in Fig. 3. Here a cDNA of approximately 550 bp whose expression is suppressed by hsp27 overexpression is denoted with an arrow. We have identified three cDNA's whose expression is suppressed in the hsp27-overexpressing transfectants. We are currently sequencing these cDNA's and confirming their respective patterns of either induction or

supression in the hsp27 transfectants using Northern blot analysis coupled with semiquantitative RT/PCR. We have extensive experience using these methods on another DD project ongoing in the laboratory and expect no problems with quickly sorting through these candidate cDNA's to then begin the arduous task of determining what role they play in hsp27's known effect on breast cancer progression.

Another approach we have undertaken is to examine known pathways important in the processes in which hsp27 expression has been implicated. Programmed cell death is an important determinant of a cell's response to drugs. We found that drug-induced apoptosis was inhibited in our transfectants. This is shown in Fig. 4 where we analyzed the DNA fragmentation ladder profiles of our cells. After 24 and 48 hours of cycloheximide treatment, oligosomal ladders were observed in the two control transfectants. In contrast, no ladders were observed with the two hsp27-overexpressing clones. To then determine which cell death pathway might be influenced by hsp27 expression, we first analyzed the bcl-2 family of proteins that are clearly recognized as important mediators of apoptosis. We utilized Western blot analysis to determine the levels of various family members in our transfectants. Levels of the pro-apoptotic proteins, bak (shown in Fig. 5), bcl-X_S and bax (data not shown) were unaffected by hsp27 overexpression. Levels of the survival promoter proteins, bcl-x_L, mcl_1, and bag were similarly unaffected (data not shown). We did find that the bcl-2 protein was reduced approximately 2-fold in our tranfectants. However, since apoptosis is inhibited in these cells and the bcl-2 protein is an inhibitor of apoptosis, its reduction in the transfectants is not consistent with it playing a role in the phenotype that we see. We conclude that the bcl-2 family of proteins may not be involved and are searching other known control pathways of apoptosis for clues to hsp27 effects on cell death.

Since we have recently determined that hsp27 overexpression increases the *in vitro* invasive properties of breast cancer cells (4), we have begun to examine pathways involved in cellular invasion. There is a known association between the matrix metalloproteinases and the invasive or metastatic phenotype. Our preliminary data suggests that matrix metalloproteinase 9 protein (MMP-9) is upregulated in our transfectants. This was observed using a Zymogram (shown in Fig. 6) where one can assay for the release of typeIV collagenase/gelatinase activity into the conditioned media. A control extract of MMP's was also included (+Con) in the Zymogram. We will continue to examine whether any of the MMP inhibitors are similarly affected by hsp27 expression concomittant with hsp27 overexpression.

CONCLUSION:

As is evidenced by this and last year's report, we have made significant progress toward our goal. We anticipate that we will successfully complete all of our stated Specific Aims by the end of Year 3 of this proposal. We will continue in our efforts at examining the mechanism(s) involved in hsp27 many effects on cellular resistance to drugs, on growth, and on the metastatic behavior of breast cancer cells. Finally, once we have dissected these pathways, we will attempt to interfere via targeting either the hsp27 promoter, or downstream along one of these pathways (Specific Aim 3). Our multi-targeted approach has cast a broad net; we anticipate a fruitful catch.

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FIGURE LEGENDS:

Figure 1: Stable overexpression of hsp27 in MDA-MB-231 cells using a constitutive promoter. Western blot analysis demonstrating overexpression of hsp27 in three transfecgtants as compared to two control, vector-alone transfectants.

Figure 2: Differential display analysis of the hsp27 transfectants. RNAs from two control transfectants (C) and hsp27 transfectants (T) clones 19 and 12(2), respectively, were analyzed by DD. A minus RT (-RT) control lane was included alongside a molecular weight marker shown in bp.

Figure 3: Differential display analysis of the hsp27 transfectants. RNAs from two control transfectants (C) and hsp27 transfectants (T) clones 19 and 12(2), respectively, were analyzed by DD. A minus RT (-RT) control lane was included alongside a molecular weight marker shown in bp.

Figure 4: DNA fragmentation analysis of the hsp27 transfectants. Cells were treated with cycloheximide 24 and 48 hours, or without drug. Genomic DNA was prepared and analyzed for the formation of DNA ladders using agarose gel electrophoresis. Control transfected clones are labeled "C"; the two transfected clones are labeled "T."

Figure 5: Western blot analysis of members of the bcl-2 family of apoptotic modulators. Shown are immunoblots of bak and bcl-2. Control transfected clones are labeled "C"; the two transfected clones are labeled "T."

Figure 6: Zymogram showing increased levels of MMP-9. M is the molecular weight marker lane and +Con is the positive control of MMPs. Control transfected clones are labeled "C"; the two transfected clones are labeled "T."

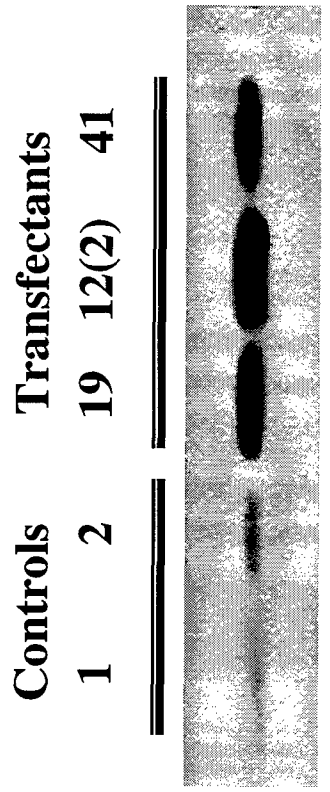


Fig. 1

Identification of cDNA's Induced by Hsp27 Overexpression

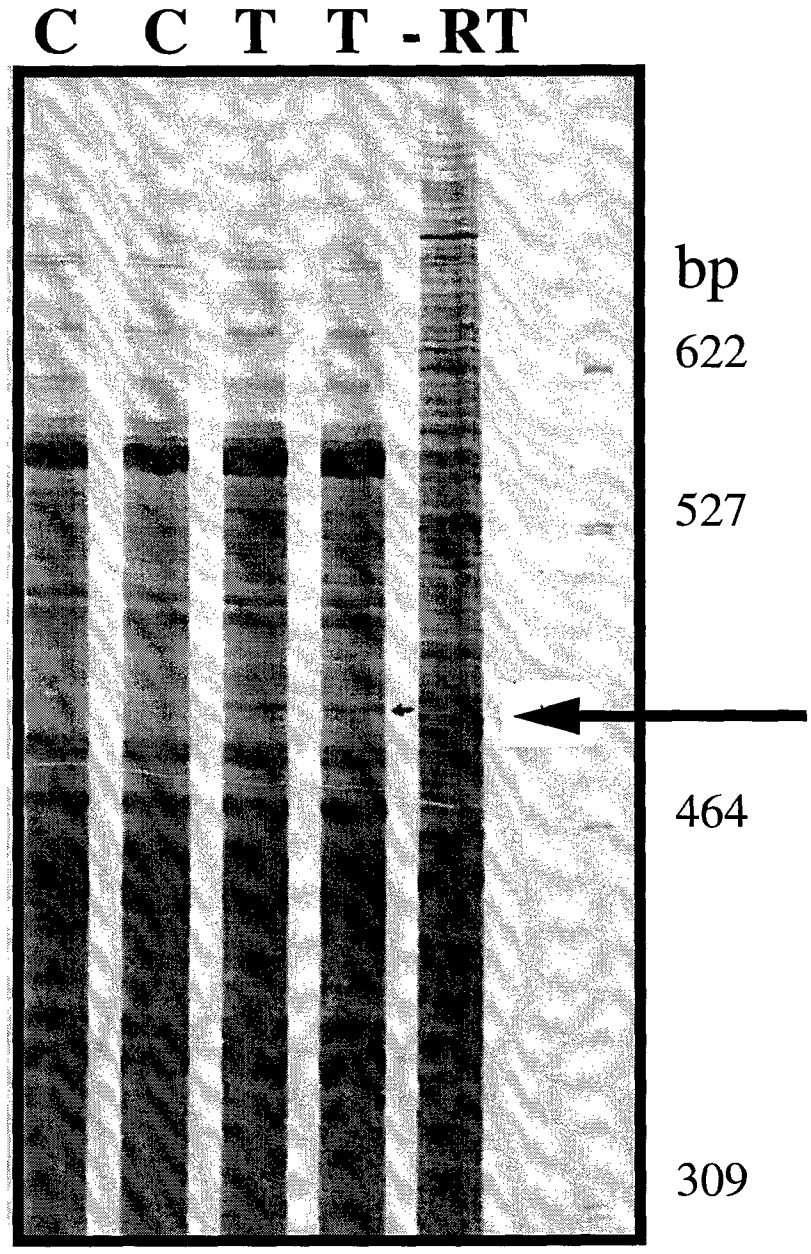


Fig. 2

Identification of cDNA's Suppressed by Hsp27 Overexpression

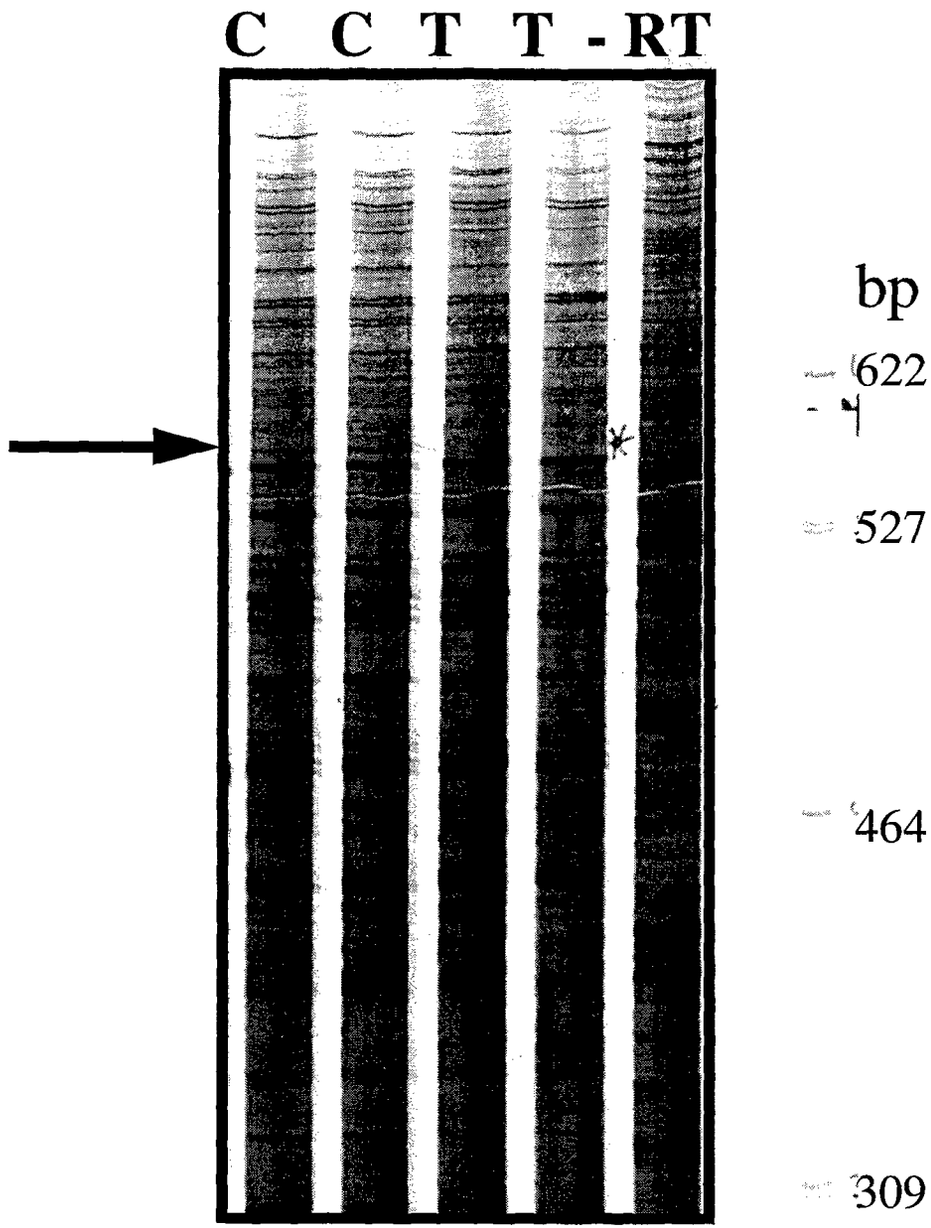


Fig. 3

Hsp27 Overexpression Inhibits Apoptosis

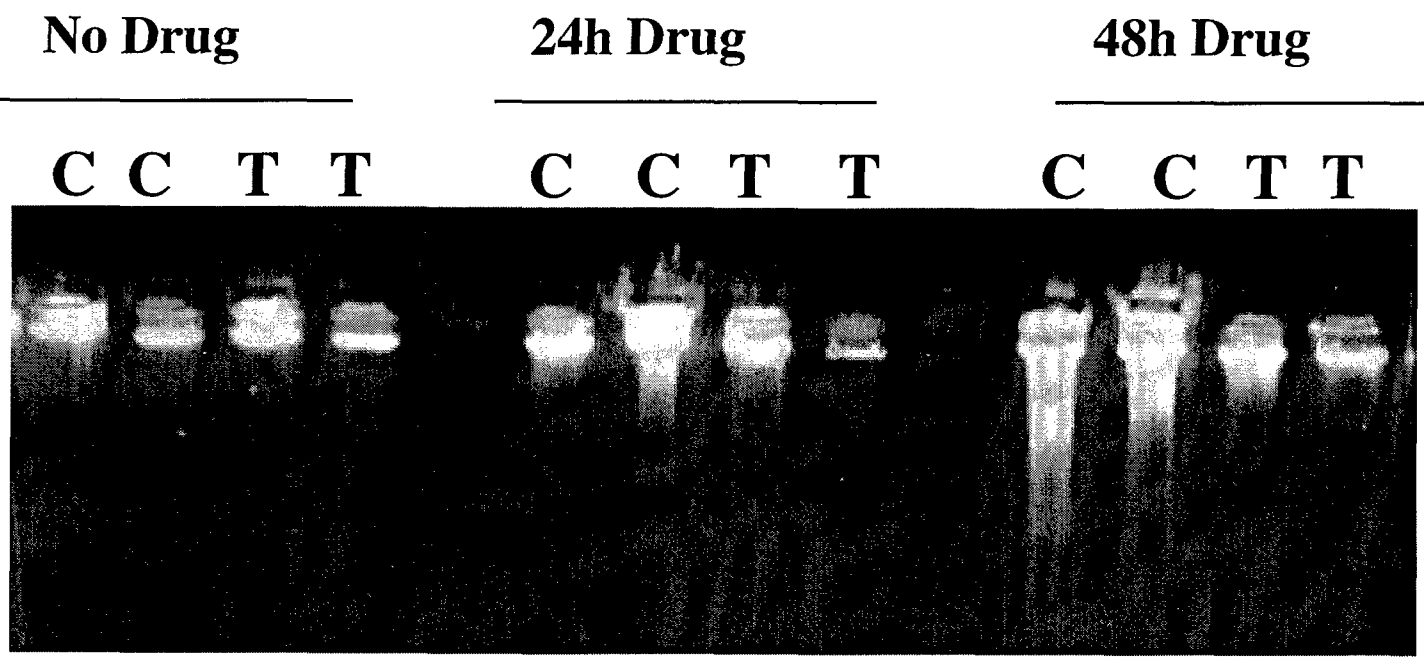
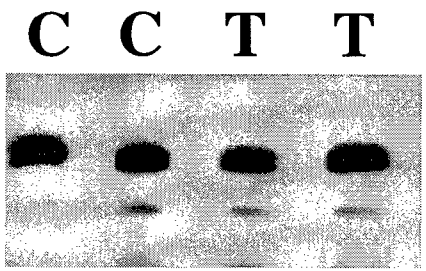
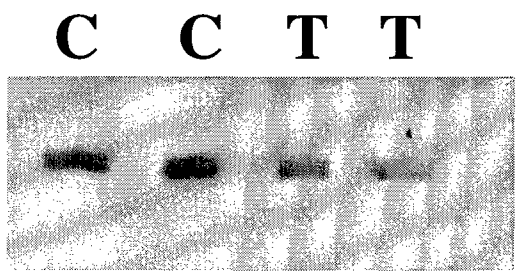


Fig. 4

The bcl-2 Apoptotic Pathway is not Involved in the Inhibition of Apoptosis by hsp27 Overexpression



bak



bcl-2

Fig. 5

Increased Levels of Active Matrix Metalloproteinase 9

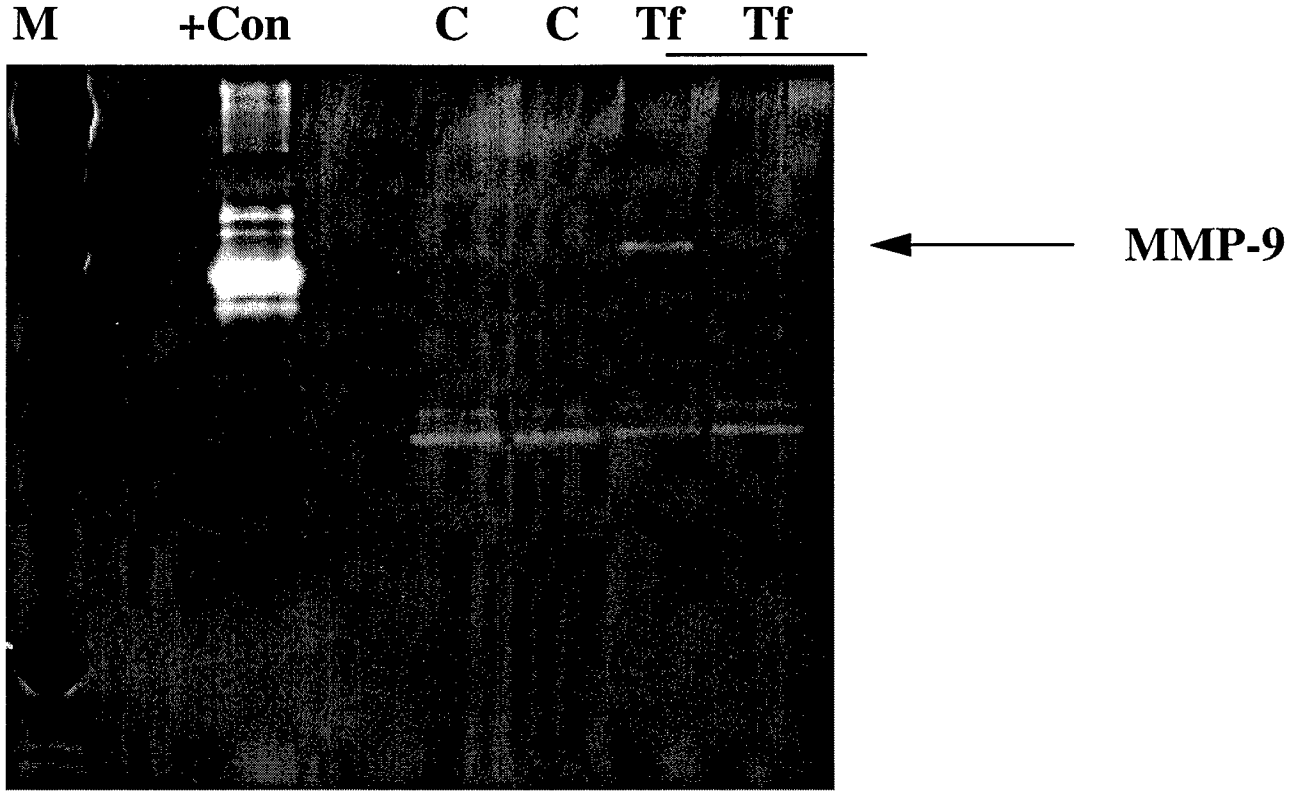


Fig. 6